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U.S. PATENT APPLICATION

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Invention: STRESSOR REGULATED GENES

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SPECIFICATION

STRESSOR REGULATED GENES

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TECHNICAL FIELD

The present invention relates, in general, to stressor-responsive genes and, in particular, to metal-responsive genes, to mRNAs, to proteins encoded therein and to uses thereof, for example, as biomonitors and in drug discovery.

BACKGROUND

The transition metal cadmium is considered to be a serious occupational and environmental toxin. Cadmium was ranked number 7 on the Agency for Toxic Substances and Disease Registry/Environmental Protection Agency "Top 20 Hazardous Substances Priority List" in 1997 (Fay et al. (1997) Food Chem. Toxicol. 34, 1163-1165). In addition, it is a frequently found contaminant at Superfund sites (Fay et al. (1997) *Food Chem. Toxicol.* 34, 1163-1165). Cadmium is used primarily in metal coatings, nickel-cadmium batteries and pigments (Friberg et al. (1986) in *Handbook of the Toxicology of Metals* (Friberg, L, Nordberg G.F. and Vouk, V., ed) pp. 130-237, Elsevier/North-Holland, Amsterdam; Aylett, B.J. (1979) in *The Chemistry, Biochemistry and Biology of Cadmium* (Webb, M., ed) pp. 1, Elsevier/North-Holland, New York). It is also continuously introduced into the atmosphere through the smelting of ores and the burning of fossil fuels (Friberg et al. (1986) in *Handbook of the Toxicology of Metals* (Friberg, L, Nordberg G.F. and

Vouk, V., ed) pp. 130-237, Elsevier/North-Holland, Amsterdam; Aylett, B.J. (1979) in *The Chemistry, Biochemistry and Biology of Cadmium* (Webb, M., ed) pp. 1, Elsevier/North-Holland, New York). It has been suggested that increased industrialization has resulted in higher levels of accumulated cadmium in humans (Fortoul et al. (1996) *Environ. Health Perspect.* 104, 630-632). The primary routes of non-occupational exposure in humans are via inhalation, and ingestion of cadmium-containing food (Waalkes et al. (1992) *Crit. Rev. Toxicol.* 22, 175-201). Humans are continuously exposed to cadmium and accumulate the metal throughout their lives in liver, lung and kidney tissue (Aylett, B.J. (1979) in *The Chemistry, Biochemistry and Biology of Cadmium* (Webb, M., ed) pp. 1, Elsevier/North-Holland, New York; Bernard et al. (1986) *Experientia Suppl.* 50, 114-123). Toxicological responses of cadmium exposure include kidney damage, respiratory diseases, such as emphysema and neurologic disorders (Waalkes et al. (1992) *Crit. Rev. Toxicol.* 22, 175-201; Chmielnicka et al. (1986) *Biol. Trace Elemants Res.* 10, 243-256). Cadmium has been classified as a type 1 human carcinogen (Internation Agency for Research on Cancer (1993) *Beryllium, Cadmium, Mercury and Exposures in the Glass Manufacturing Industry*, Vol. 58, IARC, Lyon). It induces site of exposure, lung, kidney, prostate and testicular cancers in rats and mice (Waalkes et al. (1992) *Crit. Rev. Toxicol.* 22, 175-201). Human epidemiological data suggests that it causes tumors of the male reproductive system and induces respiratory tumors (Waalkes et al. (1992) *Crit. Rev. Toxicol.* 22, 175-201; Oberdorster, G. (1986) *Scand. J. Work Environ. Health* 12, 523-537).

Intracellular damage associated with cadmium exposure includes protein denaturation, lipid peroxidation and DNA strand breaks. Proposed mechanisms by which cadmium induces this damage involve (a) metal binding to reduced cysteine residues and (b) the generation of reactive oxygen species, possibly by lowering reduced glutathione levels (Abe, T. et al. (1994) *Biochim. Biophys. Acta.* 1201, 29-36; Manca, D. et al. (1991) *Toxicology* 67, 303-323; Chin, T. A. et al. (1993) *Toxicology* 77, 145-156). To prevent cadmium-induced intracellular damage, cells respond to metal exposure by inducing the transcription of genes that encode defense and repair proteins. These proteins (a) chelate the metal to prevent further damage, (b) remove reactive oxygen species, (c) repair membrane and DNA damage and (d) renature or degrade unfolded-proteins. Cadmium has been shown to affect the steady-state levels of the mRNAs encoding metallothionein (Hamer, D.H. (1986) *Annu. Rev. Biochem.* 55, 913-951), heme oxygenase (Adam, J. et al. (1989) *J. Biol. Chem.* 264, 6371-6375), γ-glutamylcysteine synthetase (Hatcher. E.L. et al. (1995) *Free Radic. Biol. Med.* 19, 805-812), low and high molecular weight heat shock proteins (Wiegant. F.A. et al. (1994) *Toxicology* 94, 143-159) and ubiquitin (Muller-Taubenberger, A. et al. (1988) *J. Cell Sci.* 90, 51-58). In addition, increases in superoxide dismutase, catalase, glutathione peroxidase and glucose-6-phosphate dehydrogenase activities are observed following cadmium exposure in cultured cells and whole animals (Kostic, M.M. et al. (1993). *Eur. J. Haematol.* 51, 86-92; Salovsky P. et al. (1992) *Hum. Exp. Toxicol.* 11, 217-222). The mechanism(s) by which this metal modulates the levels of expression of most of these genes remains unknown.

Cadmium-activated transcription may occur through specific metal-responsive upstream regulatory elements found in the promoters of cadmium-responsive genes. These may include metal responsive element (MRE) sequences, found in most metallothionein genes (Stuart, G.W. et al. (1984) *Proc. Natl. Acad. Sci. USA* 81, 7381-7322; Searle, P.F. (1990) *Nucleic Acids Res.* 18, 4863-4690; Cizewski Culotta, V.C. et al. (1989) *Mol. Cell. Biol.* 9, 1376-1380), or cadmium-responsive elements, as found in the human heme oxygenase gene (Takeda, K. et al. (1994) *J. Biol. Chem.* 265, 14061-14064). Cadmium may also affect gene expression by influencing signal transduction pathways. Cadmium affects the activities of PKC, PKA and calmodulin (Wang, Z. et al. (1998) *J. Biol. Chem.* 273, 73-79; Beyersmann, D. et al. (1997) *Toxicol. Appl. Pharmacol.* 144, 247-261). It has been suggested that cadmium-induced transcription of the proto-oncogenes *jun* and *fos* is mediated via PKC and calmodulin (Wang, Z. et al. (1998) *J. Biol. Chem.* 273, 73-79). Thus, cadmium can modulate the activities of complex signal transduction pathways that in turn can influence the expression of a myriad of genes. However, relatively few cadmium-responsive genes have been identified. In addition, there is a paucity of information on the influence of cell-specific and developmental factors on metal-inducible gene expression.

SUMMARY OF THE INVENTION

The present invention relates to stressor-regulated genes in general and specifically to metal-regulated genes, such as cadmium-regulated genes, mRNAs and to the proteins encoded therein. The invention also relates to the use of such genes and proteins as biomonitors and in drug discovery.

Objects and advantages of the present invention will be clear from the description that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. **Confirmation of cadmium-responsive gene expression.** Total RNA was extracted from control *C. elegans* (-) and nematodes exposed to $100\mu M$ $CdCl_2$ for 24h (+) and $20\ \mu g$ was then subjected to denaturing gel electrophoresis. Northern blots were hybridized with a ^{32}P-labeled oligonucleotide probe that is specific for *mtl-2* mRNA; nt 182-221 (*upper panel*) (Freedman, J.H. et al. (1993) *J. Biol. Chem.* 268, 2554-2564). Following autoradiography, the labeled probe was removed and the membrane reprobed with a ^{32}P-labeled DNA probe specific for the myosin light-chain mRNA (*lower panel*). There are two forms of *C. elegans* myosin light-chain mRNA, containing 900 and 1300 nt (Freedman, J.H. et al. (1993) *J. Biol. Chem.* 268, 2554-2564; Cummins, C. et al. (1988) *Mol. Cell. Biol* 8, 5334-5349).

Figs. 2A and 2B. **Representative mRNA differential display band patterns of control and cadmium-treated *C. elegans*.** Total RNA was isolated from C. *elegans* exposed to cadmium for 0, 8 and 24 h and analyzed by differential display. RNA from duplicate populations of treated and control *C. elegans* was reverse transcribed and amplified with the 3'-degenerate anchored oligo(dT) primer $T_{12}MA$ and the 5'- arbitrary decamer AP-13 (Fig. 2A) and $T_{12}MG$ and RT-10 (Fig. 2B). Amplified cDNA fragments were resolved by electrophoresis in a 6% denaturing polyacrylamide gel. cDNA fragments that were subsequently isolated (DDRT15, DDRT16 and DDRT25) are indicated by *arrows*.

Figs. 3A and 3B. **Northern blot analysis showing differential expression of selected cadmium-responsive genes.** *C. elegans* poly(A^+) RNA (2μg), isolated from nematodes exposed to 100μM $CdCl_2$ for 24 h (+) or control nematodes (-), was resolved by denaturing agarose gel electrophoresis. Northern blots were hybridized with ^{32}P-labeled cDNA probes prepared from the differential display cDNA fragments VL19 (*upper panel* Fig. 3*A*) or DDRT16 (*upper panel Fig. 3B*). Following PhosphorImager analysis, the probes were removed and the membrane reprobed with a ^{32}P-labeled DNA fragment homologous to myosin light-chain mRNAs (*lower panels Figs. 3A* and *3B*).

Figs. 4A-4C. **Representative reverse-Northern dot blot of differentially expressed genes.** Cloned DNA fragments were amplified and ~100ng of the amplified product was immobilized on triplicate membranes. The membranes were then hybridized with ^{32}P-labeled cDNAs synthesized from poly(A^+) RNA prepared from either untreated *C. elegans* (Fig. 4A), or those exposed to cadmium for 8h (Fig. 4B) or 24h (Fig. 4C). The location of each differentially expressed DNA fragment, and the myosin light-chain (MLC) and metallothionein (MTL-1) controls, on the blots is presented in the following grid legend:

		Column							
		1	2	3	4	5	6	7	8
Row	A	MLC	DDRT1	DDRT2	DDRT3	DDRT4	DDRT5	DDRT6	DDRT7
	B	DDRT9	DDRT10	DDRT12	DDRT15	DDRT16	DDRT17	DDRT18	DDRT19
	C	DDRT20	DDRT21U	DDRT21D	DDRT22	DDRT23	DDRT24	DDRT25	DDRT26
	D	DDRT28	DDRT29	DDRT30	DDRT32	DDRT33U	DDRT33D	DDRT34	DDRT35
	E	DDRT36	DDRT37	DDRT38	DDRT40	DDRT41	DDRT47	DDRT48	DDRT50
	F	VL3	VL9	VL11	VL19	VL20	VL21		MTL-1

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Fig. 5. Sequences corresponding ~~to GenBank Accession Nos. shown in Table III~~. (SEQ ID NOS: 10-52 and 1-9, respectively)

DETAILED DESCRIPTION OF THE INVENTION

The present invention results, at least in part, from the realization that the non-parasitic nematode *Caenorhabditis elegans* provides an excellent model system for obtaining an integrated picture of cellular, developmental and molecular aspects of the regulation of metal-responsive gene expression (e.g., transition and heavy metal responsive gene expression, including cadmium, mercury, copper, zinc, nickel, lead, chromium, and silver responsive gene expression). The adult hermaphrodite is composed of 959 somatic cells, but contains highly differentiated muscle, nervous, digestive and reproductive systems (Sulstion, J. (1988) in *The Nematode Caenorhabditis* elegans (Wood, W. B., ed) pp. 123-155, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.; Kenyon, C. (1988) *Science* 240, 1448-1453). The developmental and cellular biology of *C.*

elegans is thoroughly understood in exceptional detail (Sulstion, J. (1988) in *The Nematode Caenorhabditis* elegans (Wood, W. B., ed) pp. 123-155, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.; Kenyon, C. (1988) *Science* 240, 1448-1453). High levels of evolutionary conservation between *C. elegans* and higher organisms are observed in many signal transduction, gene regulatory and developmental pathways (McGhee, J.D. et al. (1997) in *C. elegans II* (Riddle, D.L., Blumenthal, T., Meyer, B.J. and Priess, J.R., eds) pp. 147-184, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.; Han, M. et al. (1990) *Cell* 65, 921-931; Clark, S.G. et al. (1992) *Nature* 356, 340-344). In addition, homologues of many of the proteins induced as part of metal-activated stress-responses in vertebrates have been identified in *C. elegans*. These include metallothionein (Slice, L.W. et al. (1990) *J. Biol. Chem.* 265, 256-263; Freedman, J.H. et al. (1993) *J. Biol. Chem.* 268, 2554-2564), superoxide dismutase (Giglio, A.M. et al. (1994) *Biochem. Mol. Biol. Int.* 33, 41-44; Giglio, M.P. et al. (1994) *Biochem. Mol. Biol. Int.* 33, 37-40), ubiquitin (Zhen, M. et al. (1993) *Mol. Cell. Biol.* 13, 1371-1377; Stringham, E.G. et al. (1992) *Gene* 113, 165-173), heat shock protein 70 (Heschl, M.F.P. et al. (1989) *DNA* 8, 233-243), glutathione-S-transferase (Weston, K. et al. (1989) *Nucleic Acids Res.* 17, 2138-2139) and catalase (Ebert, R.H. et al. (1996) *Dev. Genet.* 18, 131-143). With the exception of metallothionein, the effect of cadmium on the transcription of these *C. elegans* genes remains unknown.

C. elegans also contains homologues to many of the signal transduction proteins that have been implicated in modulating the cellular/molecular response to metal exposure (Gross R.E. et al. (1990) *J. Biol. Chem.* 265, 6896-6907; Lu, X-Y. et al. (1990) *J. Biol. Chem.* 265, 3293-3303; Land, M. et al. (1994) *J. Biol. Chem.* 269, 14820-14827; Land, M. et al. (1994) *J. Biol. Chem.* 269, 9234-9244).

One of the major advantages in using *C. elegans* as a model system, for example, to identify new metal-responsive genes, is the magnitude of cDNA and genomic DNA sequence data currently available. The nematode genome is relatively small (~10^8bp), and an abundance of information is available on the genetic and physical maps of its chromosomes (Waterston, R.H. et al. in *C. elegans II* (Riddle, D.L., Blumenthal, T., Meyer, B.J. and Priess, J.R., eds) pp. 23-46, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). Currently, sequencing of the entire *C. elegans* genome is >80% completed and >50,000 ESTs have been cloned and sequenced. Megabases of genomic and cDNA sequence data are readily available through GenBank, the *C. elegans* Genome Project (Coulson, A. (1996) *Biochem. Soc. Trans.* 24, 289-291) and the *C. elegans* cDNA Sequencing Project (Sequence data and information about the *C. elegans* DNA Project can be obtained at http://www.ddbj.nig.ac.jp/c-elegans/html/CE_INDEX.html/).

As described in the Examples that follow, fifty-three differentially expressed DNA fragments from a mixed-stage population (i.e., a population at all stages of development) of cadmium-exposed *C. elegans* have been identified. Subsequent analysis confirms that the steady-state level of expression of forty-eight of these clones increases 2-6-fold following cadmium exposure. In addition, a single clone was isolated the level of expression of which decreased ~2-fold. Sequence analysis has identified *C. elegans* cosmids, predicted structural genes and ESTs that are identical to the differentially expressed mRNAs. Furthermore, the cadmium-responsive cDNAs are the products of thirty-two independent genes.

With the information provided in the Examples, three types of products, each of which is within the scope of the invention, can be directly obtained:

1. *C. elegans* genes the transcription of which is modulated by cadmium,

2. *C. elegans* mRNAs that are encoded by such genes, and

3. *C. elegans* proteins the expression of which may be affected by cadmium, and subsequently antibodies to these proteins (monoclonal or polyclonal, and antigen binding fragments thereof). These proteins can be expected to function in cadmium detoxification and/or the repair of intra- and intercellular damage.

Based on the BLAST sequence analysis provided in the Examples, the *C. elegans* cadmium-responsive genes can be divided into three categories:

1. *C. elegans* genes that encode proteins that have been shown to be responsive to cadmium in mammals (e.g., metallothionein, pyruvate carboxylase and heat-shock

protein-70);

2. *C. elegans* genes for which mammalian homologues have been identified, but the mammalian genes, etc. have not been shown to be affected by cadmium (e.g., DNA gyrase collagen, human hypothetical protein KIAA0174 [this protein in evolutionarily conserved, it is found in rats and mice] and β-adrenergic receptor kinase); and

3. *C. elegans* genes that encode novel proteins (these predicted proteins do not have any significant homology to any protein currently in the database (e.g., DDRT16)).

Human homologues of proteins in the first two categories can be obtained easily. Using the mRNAs, gene fragments and antibodies derived from the *C. elegans* cadmium-responsive genes in the third category, homologues in higher organisms (e.g., mammals, including rates, mice and humans) of the mRNAs, genes and proteins can be obtained.

While specific reference is made in the Examples that follow to cadmium-toxicity, cadmium-response, etc., for purposes of the present invention, cadmium is functioning as an archetypical stressor. The effects seen with cadmium can be expected to occur with other transition and heavy metals (see above). In addition, other classes of chemical toxins (e.g., chemical carcinogens, oxidizing agents, polyaromatic hydrocarbons) and physical stresses (e.g., ultra-violet light, ionizing radiation, heat-shock, osmotic stress, and infectious agents), can affect the expression of these genes. Accordingly, it will be appreciated that the embodiments of the invention described below encompass stressors in addition cadmium.

Biomonitors

The invention includes within its scope biomonitor kits that can contain primers that can be used to amplify specific cadmium-responsive mRNAs in PCRs, or sequence-specific oligonucleotides for Northern blot and Rnase protection assays. Such kits can also contain antibodies specific for the responsive proteins. The kits can be used to assay levels of protein using, for example, Western blot or ELISA assays.

In accordance with this embodiment, the presence of cadmium responsive mRNAs can be determined and the levels of expression of the cadmium-responsive mRNAs or proteins measured in nematodes, indigenous species or humans in potentially contaminated environments to determine if exposure to cadmium has occurred.

In addition, the effectiveness of different therapies used to treat exposure to metals or other chemical toxins can be monitored (i.e., a diagnostic tool for measuring toxicity or stress). If the expression of these genes is found to be associated with disease states, then by monitoring the levels of the protein or mRNA the progression or remission of the disease can be followed. Further, if the expression of the cadmium-responsive genes is modulated by chemotherapeutic agents, as occurs with heat shock proteins and metallothionein, then the affect of these agents on the tumor and the patient can be monitored.

Transgenic Organisms (Plants and Animals)

The invention also includes within its scope *C. elegans* or other organisms, the genome of which has been engineered to include a cadmium-responsive gene. The gene can be modified to express a reporter protein (e.g., β-galactosidase or green fluorescent protein) in place of the normal structural gene. These organisms can be exposed to potentially contaminated environmental samples, water or dirt. The level of reporter gene expression will be proportional to the amount of contamination in the sample. These organisms, which themselves are biomonitors, can be used to measure the levels of bioavailable cadmium and determine the effectiveness of clean-up efforts.

It is expected that certain of the cadmium-responsive genes encode proteins that function in the detoxification and repair of cadmium-induced cellular damage. Over-expression of these proteins would result in the organism being resistant to metal toxicity. Transgenic plants that express the *C. elegans* cadmium-responsive proteins can be generated by controlling the expression the cadmium-responsive mRNA using plant or bacterial promoters. Plants that express these proteins can be expected to be resistant to metal toxicity and thus able to grow in contaminated environments.

There is the potential that disruption of one or more of the cadmium-responsive genes in mammals (i.e., preventing the expression of the native/functional protein) could mimic a human disease state. For example, disruption of VL19 (pyruvate carboxylase) may mimic a liver disease. A transgenic organism that functions as a disease model constitutes an important tool in the pharmaceutical and medical industries.

Drug Discovery

The ability to monitor the levels of expression of the cadmium-responsive proteins can be used in drug discovery. Drugs that modulate the expression of these proteins in humans can be expected to function as modulators of other forms of stress. Chemicals or drugs that can be used to increase the expression of the cadmium-responsive proteins can be expected to protect the organism from other stresses. (For example, a drug that increases the expression of one or more of the cadmium responsive genes may allow a patient to receive a higher dose of a chemotherapeutic drug.) A drug that inhibits the ability of cadmium to induce the expression of the mammalian homologues of the *C. elegans* genes can be expected to function in the prevention of cadmium toxicity or other stress-induced toxicities.

The invention includes within its scope drugs discovered using the methods described herein.

Pathways

The ability of cadmium to induce the transcription of the *C. elegans* genes is likely to be the result of the metal activating intracellular signaling pathways. These pathways ultimately activate transcription factors, which interact with the cadmium-responsive genes. For example, cadmium is taken up by the cell, it then binds to an intracellular receptor, this binding activates a series of protein phosphorylations and dephosphorylations and in the end the cadmium responsive gene is "turned-on." It is known that cadmium is a mammalian carcinogen and teratogen. However, the mechanisms that control these effects have not been discovered. A pathway that regulates the expression of the cadmium-responsive genes may represent new a pathway for the development of tumors or other disease. It may also define new pathways that control cell growth and differentiation.

Cadmium-responsive mRNA and protein probes that can be used in the identification of these pathways are within the scope of the invention. Such mRNA and protein probes can be used to monitor the effectiveness of drugs that interact with components of these pathways.

The non-limiting Examples that follow describe certain aspects of the invention in greater detail.

EXAMPLES

The following experimental details are referenced in the specific Examples that follow.

Growth and isolation of C. elegans – The N2 strain of *C. elegans* was grown in liquid S medium (0.1M NaCl, 50mM potassium phosphate, pH 6.0, 5μg/ml cholesterol, 10mM potassium citrate, 3mM $CaCl_2$, 3mM $MgCl_2$, 50μM EDTA, 25μM $FeSO_4$, 10μM $MnCl_2$, 10μM $ZnSO_4$ and 1μM $CuSO_4$) using *E. coli* OP50 as a food source (Brenner, S. (1974) *Genetics* 77, 71-94). In experiments where nematodes were exposed to cadmium, the medium was supplemented with 100µM $CdCl_2$ (Freedman, J.H. et al. (1993) *J. Biol. Chem.* 268, 2554-2564). *C. elegans* were grown in the presence of metal for 8h or 24h at ~20°C. Nematodes were then collected following centrifugation at 800xg for 5 min. Pellets were suspended in 50mM NaCl containing 35% sucrose (final concentration) and viable nematodes were collected from the top of the solution following centrifugation at 1000xg for 5 min at 4°C. Nematodes were then washed three times by suspension in M9 buffer (22mM KH_2PO_4, 42mM Na_2HPO_4, 85mM NaCl, 1mM $MgSO_4$) followed by sedimentation at 800xg. Washed nematode pellets were finally suspended in a small volume of M9 buffer, rapidly frozen in liquid nitrogen and stored at -80°C.

RNA isolation — Total RNA was isolated from mix-stage populations of *C. elegans* exposed to 100µM $CdCl_2$ for 8h and 24h and control, non-exposed nematodes. Frozen worms were first ground into a fine powder using a liquid nitrogen-cooled mortar and pestle. Powdered *C. elegans* (200 mg) were then homogenized in 2ml of TRIzol (GIBCO/BRL). RNA was then collected from the aqueous phase following the addition of chloroform, precipitated by adding isopropyl alcohol and then air-dried. The dried RNA pellet was then dissolved in diethyl pyrocarbonate (DEPC)-treated water. For some experiments, poly(A^+) RNA was subsequently isolated using the Poly(A) Tract System following manufacturer's instructions (Promega).

mRNA Differential display — Differential display was performed following the protocol of Liang and Pardee (Liang, P. et al. (1992) *Science* 257, 967-971). Briefly, 50μg of total RNA isolated from either of three populations of *C. elegans*, controls or those grown in the presence of cadmium for 8h or 24h, was treated with 10 units of RNase-free DNase I (Boehringer Mannheim) in 10mM Tris-Cl buffer, pH 8.3, containing 50mM KCl and 1.5mM $MgCl_2$. The DNA-free RNA was precipitated with ethanol and dissolved in DEPC-treated water. First-strand cDNAs were generated in reverse transcriptase reactions containing 0.2μg DNA-free total RNA, reverse transcriptase buffer (25mM Tris-Cl, pH 8.3, 38mM KCl, 1.5mM $MgCl_2$, 5mM dithiothreitol), 5μM of each dNTP and 1μM of one of four 3'-degenerate anchored oligo(dT) primers. The 3'-degenerate anchored oligo(dT) primers have the sequence: $T_{12}MG$, $T_{12}MA$, $T_{12}MT$, or $T_{12}MC$, where M is 3-fold degenerate for G, A, and C. Primers were annealed to the RNA template by incubating the reaction mixture for 5 min at 65°C, then for 10 min at 37°C. First strand cDNA synthesis was achieved following the addition of 100 units of Moloney murine leukemia virus reverse transcriptase (GIBCO/BRL) and incubating at 37°C for 50 min. The reaction was terminated by heating at 95°C for 5 min, which inactivates the reverse transcriptase.

Amplification of cDNA fragments was performed in 20μl reactions. Each PCR mixture contained 2μl of the products from one of the four above reverse transcriptase reactions and 18μl of a solution containing *Taq*-PCR buffer (10mM Tris-Cl, pH 8.4, 50mM KCl, 1.5mM $MgCl_2$, 0.01% gelatin), 1μM of the same 3'-

degenerate anchored oligo(dT) primer used in the first-strand synthesis reaction, four dNTPs (2µM each), 10µCi [α-^{35}S]dATP (Amersham), 1 unit AmpliTaq DNA polymerase (Perkin-Elmer) and 0.2µM of one of twenty 5'-arbitrary decamers. The sequences of the 5' arbitrary primer used in these reactions are presented in Table I. Reaction mixtures were subjected to 40 cycles of the PCR using the following parameters: denature at 94°C for 30 seconds, anneal at 42°C for 2 min, elongate at 72°C for 30 seconds. All PCRs were performed in duplicate. The amplified cDNAs produced from duplicate reactions of RNA isolated from control, 8h-treated and 24h-treated *C. elegans* were size fractionated in parallel by polyacrylamide gel electrophoresis in 6% acrylamide/8M urea gels.

Table I

Sequences of the 5′-Arbitrary Decamer Primers used in Differential Display

Primer Designation	Sequence
AP-3	AGGTGACCGT (SEQ ID NO:53)
AP-4	GGTACTCCAC (SEQ ID NO:54)
AP-6	GCAATCGATC (SEQ ID NO:55)
AP-7	CCGAAGGAAT (SEQ ID NO:56)
AP-8	GGATTGTGCG (SEQ ID NO:57)
AP-9	CGTGGCAATA (SEQ ID NO:58)
AP-10	TAGCAAGTGC (SEQ ID NO:59)
AP-13	AGTTAGGCAC (SEQ ID NO:60)
AP-15	AGGGCCTGTT (SEQ ID NO:61)
AP-18	CTGAGCTAGG (SEQ ID NO:62)
RT-1	TACAACGAGG (SEQ ID NO:63)
RT-2	TGGATTGGTC (SEQ ID NO:64)
RT-3	CTTTCTACCC (SEQ ID NO:65)
RT-4	TTTTGGCTCC (SEQ ID NO:66)
RT-5	GGAACCAATC (SEQ ID NO:67)
RT-6	AAACTCCGTC (SEQ ID NO:68)
RT-7	TCGATACAGG (SEQ ID NO:69)
RT-8	TGGTAAAGGG (SEQ ID NO:70)
RT-9	TCGGTCATAG (SEQ ID NO:71)
RT-10	GGTACTAAGC (SEQ ID NO:72)

Following electrophoresis, gels were dried onto Whatman 3MM paper and exposed to Kodak X-AR film for 24h. Differentially expresses cDNAs were visualized by autoradiography. To isolate differentially expressed cDNA fragments, regions of dried gels corresponding to the cDNAs were excised. Gel slices were rehydrated in 100µl dH_2O following a 10-min incubation at room temperature. The cDNA was then extracted from the rehydrated gels by incubating at 100°C for 15 min in tightly capped microcentrifuge tubes. cDNA was recovered by ethanol precipitation in the presence of 0.3M sodium acetate and 50µg of glycogen (Boehringer Mannheim). The eluted cDNA was reamplified in a 40µl reaction with the identical pair of primers used in the mRNA differential display reaction. PCR reaction conditions were similar to those above except, the concentration of the dNTPs was increased to 20µM and the [α-^{35}S]dATP was omitted. Amplified cDNA fragments were resolved by gel electrophoresis using a 1.5% agarose gel and then purified using QIAEXII kits (QIAGEN).

Subcloning and DNA sequence analysis — Gel-purified cDNAs were directly inserted into the T-A cloning vector pGEM-T (Promega). DNA inserts were subsequently sequenced using T7 and SP6 primers by the dideoxynucleotide chain termination procedures of Sanger et. al (Sanger, F. et al. (1977) *Proc. Natl. Acad. Sci. U.S.A.* 74, 5463-5467) (United States Biochemicals Sequenase Kit, Version 2.0).

Computer analysis — Analysis of cDNA sequence data including sequence comparisons, alignments and generation of contigs were performed using PC/GENE-Intelli-Genetics software. BLAST analysis (Altschul, S.F. et al. (1990) *J. Mol. Biol.* 215, 403-410) was carried out through the National Center for Biotechnology Information and the *C. elegans* Genome Project Internet servers using the non-redundant, *C. elegans* genome and *C. elegans* EST databases. For some sequence analysis the "A *C. elegans* database" (ACeDB) software was used (Eeckman, F.H. et al. (1995) *Methods Cell. Biol.* 48, 583-605). Predicted *C. elegans* genes were identified by the *C. elegans* Genome Project using the GENEFINDER program (Favello, A. et al. (1995) *Methods Cell. Biol.* 48, 551-569).

Northern Blot Analysis—Samples of total RNA (20µg) or poly(A^+) RNA (2µg) were denatured in a 2.2M formaldehyde/50% (v/v) formamide buffer and then subjected to denaturing gel electrophoresis on a 1.5% agarose/2.2M formaldehyde gel. Size-fractionated RNAs were then transferred to Nytran membrane (Schleicher and Schuell). Membranes were probed with ^{32}P-labeled cDNA fragments of the differentially expressed mRNAs. cDNAs to be used as probes were generated by the PCR from the cloned DNA fragments recovered from differential display gels. cDNAs were labeled with [α-^{32}P]dCTP (Amersham) by random-primed labeling. Membranes were hybridized in 6 X SSC (1 X SSC = 0.15M sodium chloride, 15mM sodium citrate, pH 7.0), 1.25x Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), 300ng denatured sonicated salmon sperm DNA and heat-denatured probe at 42°C for 16h. Following hybridization, membranes were washed at a high stringency of 50°C for 30 min in 0.1 X SSC/0.1% SDS. The amount of probe hybridizing to the RNA was determined by PhosphorImager analysis (Molecular Dynamic System). After images were obtained, membranes were incubated at 95°C for 1h in 0.1% SDS to remove the bound probe. They were then hybridized with a ^{32}P-labeled *C. elegans* myosin light-chain probe, which served as a loading control (Freedman, J.H. et al. (1993) *J. Biol. Chem.* 268, 2554-2564). As a positive control, membranes were also hybridized to a ^{32}P-labeled *C. elegans* metallothonein-2 (*mtl*-2) cDNA probe (Freedman, J.H. et al. (1993) *J. Biol. Chem.* 268, 2554-2564). Quantification of radioactivity was performed using the ImageQuant program (Molecular Dynamic System). Steady-state levels of mRNA expression were all normalized to that of the constitutively expressed myosin light-chain mRNAs (Freedman, J.H. et al. (1993) *J. Biol. Chem.* 268, 2554-2564; Cummins, C. et al. (1988) *Mol. Cell. Biol* 8, 5334-5349).

Reverse-Northern dot-blot analysis — Changes in the steady-state levels of differentially expressed mRNAs in *C. elegans* following cadmium-exposure were also determined by reverse-Northern dot-blot analysis by the modified procedure of Zhang et al. (Cummins, C. et al. (1988) *Mol. Cell. Biol* 8, 5334-5349). Briefly, differentially expressed cDNAs that were previously cloned into pGEM-T were amplified using primers that anneal to the T7 and SP6 RNA polymerase binding sites, which flank the cDNA insert. cDNAs were amplified and subsequently purified using a PCR-spin column (QIAGEN). Approximately 100ng of each amplified cDNA were denatured by mixing with 0.1N NaOH (final concentration) and incubating at 100°C for 5 min. The solution was neutralized following the addition of 3 X SSC (final concentration) and then the volume adjusted to 700 µl with dH_2O. 200-µl of each sample was applied to one of three Nytran membranes in a Bio-Dot microfiltration apparatus (BioRad). Membranes were then baked for 30 min at 80°C under vacuum. As positive and loading controls 100ng of *mtl*-1 cDNA and myosin light chain DNA were also applied to each membrane, respectively.

Three pools of single-stranded ^{32}P-labeled cDNA probes were prepared from poly(A^+) RNA isolated from control, 8h and 24h cadmium-treated nematodes. cDNAs were generated from a mixture of mRNAs in a 25µl reverse transcriptase reactions which contained 2µg poly(A^+) RNA, 1µg oligo$(dT)_{18}$ primer, reverse transcriptase buffer, 800µM dATP, dGTP and dTTP, 4.5µM dCTP, 100µCi [α-^{32}P]dCTP (3000Ci/mmol), 20 units RNase inhibitor and 200 units Moloney murine leukemia virus reverse transcriptase. The reaction mixture was incubated at 37°C for 1h, then at 95°C for 5 min to terminate the reaction. Unincorporated nucleotides were separated from the labeled cDNAs by using a G-25 spin column (Boehringer Mannheim). Equal amounts (5×10^6 cpm/ml) of each radioactive cDNA mixture were heat-denatured and then hybridized separately to one of the three membranes at 42°C for 16h in hybridization buffer. Membranes were washed at a high stringency of 0.1 X SSC, 0.1% SDS at 55°C for 30 min. The amount of ^{32}P-labeled probe bound to each differentially expressed cDNA was quantified by PhosphorImager analysis and levels of expression of the cognate mRNAs normalized to that of the myosin light-chain mRNA.

EXAMPLE 1

Effect of Cadmium on Gene Expression

The level of the *C. elegans mtl-2* mRNA was measured by Northern blot analysis to confirm that the cadmium-exposure protocol outlined above affects gene expression (Freedman, J.H. et al. (1993) *J. Biol. Chem.* 268, 2554-2564). A ^{32}P-labeled oligonucleotide probe that is specific for the 3'-end of the *mtl-2* mRNA was hybridized to a membrane that contained RNA prepared from control *C. elegans* or nematodes exposed to 100μM $CdCl_2$ for 24h (Fig. 1). The steady-state level of *mtl-2* mRNA increased in response to cadmium exposure to that previously reported (Freedman, J.H. et al. (1993) *J. Biol. Chem.* 268, 2554-2564). This verified that the cadmium-treatment protocol alters gene expression in *C. elegans* and can be used for the differential display analysis.

EXAMPLE 2

Identification of Cadmium-responsive Genes by Differential Display

mRNA expression patterns of non-treated *C. elegans* and those exposed to cadmium for 8h and 24h were compared by mRNA differential display in order to identify new genes whose transcription is regulated by cadmium. A total of twenty 5'-arbitrary decamers, including five that have sequences that are homologous to the *mtl-1* cDNA, were used. Each of the twenty decamers was paired with one of four 3'-degenerate anchored oligo(dT) primers and used to amplify cDNAs prepared from control and cadmium-treated *C. elegans*. All amplification experiments were performed in duplicate using RNA prepared from independently treated populations of *C. elegans*. This generated a total of 480 separate reactions: three populations of cDNA amplified using eighty combinations of primers, in duplicate.

To bias against isolating differentially displayed cDNAs that are "false positive" (Liang, P. (1993) *Nucleic Acids Res.* 21, 3269-3275), only cDNAs whose level of expression were affected by cadmium in duplicate experiments were selected for further analysis. In addition, cDNA fragments that have altered levels of expression in both the 8h and 24h cadmium-treated samples were selected. Representative data is presented in Fig. 2. A total of seventy-five differentially expressed cDNA fragments were identified and excised from the gels. Of the cDNAs that were selected for further evaluation, the majority showed an increase in band intensity as a result of cadmium treatment, compared to identical sized DNA fragments from the control sample. A single product, VL9, was identified that showed decreased band intensity in the cadmium-treated *C. elegans* cDNA compared to the control.

Fifty-three cDNAs were successfully extracted from the acrylamide gels, reamplified and cloned. Because of the large number of cDNAs successfully isolated, no further attempt was made to clone the remaining fragments. The cloned cDNA fragments ranged in size 141bp to 326bp (Table II). These cDNAs were subsequently sequenced, and changes in the *in vivo* steady-state level of expression of the cognate mRNAs following cadmium treatment evaluated.

Table II

Changes in the steady-state level of differentially expressed mRNAs

Clone name	Primers used in PCR	Size of PCR product (bp)	Fold-change in mRNA levels following cadmium exposure[a]	
			8 hours	24 hours
DDRT1	T_{12}MG/RT-4	255	2.2	3.2
DDRT2●[b]	T_{12}MG/RT-4	214	3.1	3.9
DDRT3▲	T_{12}MC/RT-5	162	2.0	2.4
DDRT4▲	T_{12}MC/RT-5	187	2.5	2.8
DDRT5	T_{12}MA/RT-5	254	3.2	3.9
DDRT6	T_{12}MC/RT-5	219	4.7	5.0
DDRT7●	T_{12}MG/RT-6	217	3.6	4.0
DDRT9	T_{12}MA/RT-6	240	3.5	2.8
DDRT10	T_{12}MC/RT-7	212	2.7	2.5
DDRT12	T_{12}MA/RT-7	226	3.0	3.2
DDRT15	T_{12}MG/RT-10	248	2.8	3.1
DDRT16●	T_{12}MG/RT-10	240	4.3	4.9 (3.5)[c]
DDRT17	T_{12}MA/RT-10	200	3.7	5.7
DDRT18	T_{12}MC/RT-10	228	2.2	2.9
DDRT19■	T_{12}MT/AP-3	322	2.7	3.9
DDRT20■	T_{12}MT/AP-3	213	2.6	2.8
DDRT21U	T_{12}MC/AP-4	284	2.2	2.6
DDRT21D	T_{12}MC/AP-4	188	2.2	4.2
DDRT22	T_{12}MC/AP-4	292	2.5	3.3
DDRT23▼	T_{12}MG/AP-4	277	3.3	3.0
DDRT24	T_{12}MG/AP-4	272	1.3	1.5
DDRT25◆	T_{12}MA/AP-13	228	3.9	4.4
DDRT26●	T_{12}MG/AP-13	238	4.4	4.7
DDRT28◆	T_{12}MA/AP-13	308	1.7	1.8
DDRT29	T_{12}MG/AP-15	141	3.9	4.4
DDRT30	T_{12}MA/AP-15	289	2.7	3.1
DDRT32▼	T_{12}MT/AP-15	252	2.6	2.4
DDRT33U	T_{12}MG/AP-18	208	3.0	3.5
DDRT33D	T_{12}MG/AP-18	171	3.1	3.5
DDRT34	T_{12}MA/AP-18	189	2.4	3.0
DDRT35	T_{12}MT/AP-18	314	1.6	1.9
DDRT36✚	T_{12}MC/AP-18	292	1.1	1.1
DDRT37	T_{12}MC/AP-4	267	1.0	1.2
DDRT38▼	T_{12}MC/AP-4	238	1.1	1.8
DDRT40	T_{12}MA/AP-8	264	1.9	2.3
DDRT41	T_{12}MA/AP-8	154	2.2	2.1

DDRT47✚	T_{12}MC/AP-13	165	1.5	1.4
DDRT48▼	T_{12}MG/AP-13	232	2.1	2.6
DDRT50	T_{12}MT/AP-15	254	2.2	2.2
VL1★	T_{12}MT/AP-6	217	(4.7)	(5.2)
VL3	T_{12}MT/AP-6	199	1.4	2.4
VL5★	T_{12}MT/AP-7	216	ND[c]	ND
VL7★	T_{12}MT/AP-7	217	ND	ND
VL8★	T_{12}MC/AP-6	214	ND	ND
VL9	T_{12}MT/AP-6	356	-1.7	-2.3
VL10★	T_{12}MT/AP-7	196	ND	ND
VL11	T_{12}MT/AP-7	142	3.2	6.1
VL12★	T_{12}MG/AP-10	216	ND	5.2
VL13★	T_{12}MG/AP-9	216	ND	ND
VL15★	T_{12}MT/AP-10	217	ND	ND
VL19	T_{12}MG/AP-9	285	3.6	5.0 (2.3)
VL20	T_{12}MT/AP-9	148	2.8	5.0
VL21	T_{12}MC/AP-10	326	2.2	2.5 (2.0)

[a] Steady-state levels of differential expressed RNAs were determined by reverse-Northern blot analysis. Fold-change in expression is relative to non-exposed *C. elegans*. All values have been normalized to the level of myosin light chain mRNA and are the average of three independent experiments.

[b] Clones labeled with identical symbols (●■▼◆▲★✚) indicate that the differentially expressed cDNAs are derived from the same gene (see Table III).

[c] Values in *parenthesis* were determined by Northern blot analysis.

[d] ND; Not determined.

EXAMPLE 3

Northern and Reverse-Northern Blot Analyses with Differentially Displayed cDNA Fragments

Northern blot analysis was initially used to confirm that the differentially expressed cDNA fragments, VL3, VL9, VL11, VL19, VL20, VL21 and DDRT16, represent mRNAs whose steady-state levels change following cadmium exposure *in vivo*. RNA blots were prepared with either size fractionated total RNA or poly(A^+) mRNA, which were isolated from control and cadmium exposed *C. elegans*, and probed with ^{32}P-labeled cDNA fragments. Northern blot analysis confirmed that as a result of cadmium treatment, the levels of expression of VL19 and DDRT16 increase 2.3-fold and 3.5-fold, respectively (Fig. 3). There is also a 2-fold increase in the level of VL21 mRNA. The mRNAs for VL3, VL9, VL11 and VL20 were not detected by Northern blots containing poly(A^+) mRNA isolated from either control or cadmium-treated *C. elegans*.

Reverse-Northern dot-blot analysis (Zheng, H. et al. (1996) *Nucleic Acids Res.* 24, 2454-2455) was performed as an alternative to traditional Northern blots. In this analysis, all of the differentially expressed clones as well as positive and loading controls were simultaneously examined. Representative results are shown in Fig. 4, and Table II summarizes the quantitative analysis from three separate experiments.

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C. elegans mtl-1 and myosin light chain DNAs were used as controls in the reverse-Northern dot-blot analysis. The *mtl-1* mRNA is induced ~5-fold after cadmium treatment (Fig. 4), while the level of expression of myosin light-chain mRNA remained constant. These results are consistent with those previously reported (Freedman, J.H. et al. (1993) *J. Biol. Chem.* 268, 2554-2564).

Of the fifty-three cDNA fragment isolated, forty-six of the clones corresponded to *C. elegans* RNAs whose level of expression increased ~2- to 5-fold following an 8-h cadmium-treatment, and up to 6-fold after a 24-h exposure (Table II). Two differentially expressed mRNAs, VL3 and DDRT38, do not show any significant change in expression following an 8h cadmium exposure, compared to control nematodes. After a 24h exposure, however, there is an ~2-fold increase in their levels of expression. The level of expression for VL9 decreased ~2-fold in response to cadmium. These results confirm that the changes in the levels RNA observed by differential display analysis reflect the *in vivo* molecular response of *C. elegans* to cadmium. Four of the clones, DDRT24, DDRT36, DDRT37 and DDRT47, did not significantly change their level of expression after either 8h or 24h cadmium exposure (Table II). These clones are defined as false positives.

EXAMPLE 4

Nucleotide Sequencing and Homology Searching

The nucleotide sequences of the differentially expressed DNA fragments were compared against the *C. elegans* genomic and EST databases. Only seven of the cDNA fragments, DDRT12, DDRT15, DDRT21D, DDRT29, DDRT41, VL3 and VL21 did not show a >95% sequence identity to regions of the *C. elegans* genome (Table III). Forty-four clones were identical to *C. elegans* cosmid and/or yeast artificial chromosome (YAC) sequences. In addition, thirty-nine clones were identical to *C. elegans* ESTs (26 of 39) or predicted genes (32 of 39).

Table III

Sequence analysis of cadmium-regulated, differentially expressed cDNAs

lone name	Sequence Identity/Homology[a]		GenBank accession no.[h]
	Cosmid[b]	Gene product[c]	
DDRT1	T09B4	T09B4.1,CELK00886	AF071359
DDRT2●[d]	F35E8	F35E8.11	AF071362
DDRT3▲	F35E12	F35E12.7	AF071382
DDRT4▲	F35E12	F35E12.7	AF071391
DDRT5	C56C10	C56C10.12, CELK05910	AF071396
DDRT6	W03C9	W03C9.5, CELK06396	AF071397
DDRT7●	F35E8	F35E8.11	AF071398
DDRT9	C35D10	ND[e]	AF071399
DDRT10	C49C3	ND	AF071353
DDRT12	ND	ND	AF071354
DDRT15	ND	ND	AF071355
DDRT16●	F35E8	F35E8.11	AF071356
DDRT17	C49A9	C49A9.4, CELK02276	AF071358
DDRT18	F13G4	F13G3.4, CELK06645	AF071360
DDRT19■	ZK849	ND	AF071361
DDRT20■	ZK849	ND	AF071363
DDRT21U	Y111B2[f]	ND	AF072438
DDRT21D	ND	CELK05123	AF071364
DDRT22	F57G9	ND	AF071365
DDRT23▼	F31C3	*C. elegans* rDNA tandem repeats	AF071376
DDRT24	C56C10	C56C10.8; CELK02788; Human transcription factor BTF3[g]	AF071377
DDRT25◆	R119	R119.5; CELK00686	AF071378

DDRT26●	F35E8	F35E8.11	AF071379
DDRT28◆	R119	R119.5; CELK00686	AF071380
DDRT29	ND	ND	AF071138
DDRT30	C27H5	C27H5.5; CELK02088, *C. elegans* collagen (*col-36*)	AF071383
DDRT32▼	F31C3	*C. elegans* rDNA tandem repeats	AF071384
DDRT33D	C34F6	CELK01885; *C. elegans* cuticle collagen	AF071385
DDRT34	F20C5	F20C5.1; CELK01295	AF071386
DDRT35	R11D1	R11D1.1; CELK02809; Human hypothetical protein KIAA0174	AF071387
DDRT36✚	D2096	D2096.8; CELK01725; Human nucleosome assembly protein1 LIKE-1	AF071388
DDRT37	K11H12	K11H12.2; CELK02043; Rat 60S ribosomal protein	AF071389
DDRT38▼	F31C3	*C. elegans* rDNA tandem repeats	AF071390
DDRT40	W02B3	W02B3.2; Bovine β-adrenergic receptor kinase	AF071392
DDRT41	ND	*Spiroplasma citr* DNA gyrase subunit B	AF071393
DDRT47✚	D2096	D2096.8; CELK01725; Human nucleosome assembly protein1 LIKE-1	AF071394
DDRT48▼	F31C3	*C. elegans* rDNA tandem repeats	AF071395
VL1★	K11G9	K11G9.5; CELK3309; *C .elegans* metallothionein-1*(mtl-1)*	AF073166
VL3	ND	ND	AF071374
VL5★	K11G9	K11G9.5; CELK3309; *C .elegans* metallothionein-1*(mtl-1)*	AF071375
VL7★	K11G9	K11G9.5; CELK3309; *C .elegans* metallothionein-1*(mtl-1)*	AF072436
VL8★	K11G9	K11G9.5; CELK3309; *C .elegans* metallothionein-1*(mtl-1)*	AF072437
VL9	C50B6	ND	AF071375
VL10	K11G9	K11G9.5; CELK3309; *C .elegans* metallothionein-1*(mtl-1)*	AF073167
VL11	C06G3	C60G3.8	AF073168
VL12★	K11G9	K11G9.5; CELK3309; *C .elegans* metallothionein-1*(mtl-1)*	AF072434
VL13★	K11G9	K11G9.5; CELK3309; *C .elegans* metallothionein-1*(mtl-1)*	AF073169
VL15★	K11G9	K11G9.5; CELK3309; *C .elegans* metallothionein-1*(mtl-1)*	AF072435
VL19	D2023	D2023.2; CELK00011;Human pyruvate carboxylase	AF073170
VL20	B0228	B0228.1	AF071371
VL21	--	CELK00200; *C. elegans* mitochondrial hsp70 protein F precursor	AF071372

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[a] Analyzed with BLASTN using GenBank and *C. elegans*-specific databases. The sequences have >80% nucleotide sequence identity.

[b] *C .elegans* genomic cosmids that have >90% nucleotide sequence identity.

[c] Predicted genes are designated by the cosmid name followed by the structural gene number (e.g., F35E8.11). *C. elegans* ESTs are denoted with the "CELK" designation.

[d] Clones labeled with identical symbols (●■▼◆▲★✚) indicate that the differentially expressed cDNAs are derived from the same gene.

[e] ND, Not detected.

[f] Yeast Artificial Chromosome

[g] Homologous proteins are presented that have a >60% amino acid sequence identity, based on BLASTX analysis.

[h] See Fig. 5

The results of the BLASTN analysis showed that the differentially expressed cDNA fragments were derived from thirty-two independent genes (Table III). Eight cDNA clones, VL1, VL5, VL7, VL8, VL10, VL12, VL13 and VL15, are identical to the *mtl-1* cDNA sequence. This result is not unexpected because five of the 5'-random decamer primers used in the amplification reactions are identical, or have a one-nt mismatch, to regions in the *mtl-1* cDNA. These primers were specifically selected to function as internal controls which amplified the *mtl-1* cDNA, in order to confirm the efficacy of differential display analysis in identifying cadmium-responsive *C. elegans* genes.

Four clones, DDRT2, DDRT7, DDRT16 and DDRT26, are derived from the predicted gene F35E8.11. They were amplified using the same 3'-degenerate oligo(dT) primer, however, four different 5'-primers were used (Table II). In several cases, pairs of cDNA fragments were isolated that are products of the same gene, DDRT3 and DDRT4; DDRT19 and DDRT20; and DDRT25 and DDRT28. The lengths of the cDNA fragments in each pair are different. Each pair of cDNA products was, however, amplified using identical pair of primers (Table II). Four cDNA fragments were isolated that have sequences that are homologous to the *C. elegans* rDNA tandem repeats in cosmid F31C3. The sequences of two of the clones, DDRT23 and DDRT38, are identical. The sequences of these rDNAs are homologous to a region in the cosmid between nt 25265 and 25433. The sequences of clones DDRT48 and DDRT32 are not homologous to the other rDNA clones. They are identical to regions of the cosmid approximately 3kb from the region homologous to DDRT23 and DDRT38, nt 28041-28245 and nt 28634-28887, respectively.

cDNA fragments isolated using the differential display technique typically contain 3'-untranslated regions of the mRNAs. In order to identify proteins that are encoded by these mRNAs, we took advantage of *C. elegans* cDNA Project data.

The nucleotide sequences of many of the cadmium-responsive mRNAs are identical to cDNA clones isolated and sequenced by the Project. By assembling contigs consisting of the differentially expressed cDNA sequence and the related *C. elegans* EST sequences, longer open-reading frames were generated. For example, the differentially expressed clone DDRT33D is 171bp and its sequence is 100% identical to the 3'-end of the *C. elegans* clone yk58b1. This clone is a member of a group of six related cDNA clones (cDNA group: CELK01885) for which 3' and 5' sequence data is available. This EST data was collected and assembled into a single 880bp contig that was analyzed by BLASTX (the clones within the CELK01885 cDNA group are yk364f10 (GenBank Accession Number C69593 and GenBank Accession Number C58303), yk279e3 (GenBank Accession Number C68110 and GenBank Accession Number C57084), yk363b2 (GenBank Accession Number C69396 and GenBank Accession Number C58214), yk146f5 (GenBank Accession Number C10425), yk92a12 (GenBank Accession Number D66109 and GenBank Accession Number D69777) and yk58b1 (GenBank Accession Number D65495 and GenBank Accession Number D68941)). This protocol was used for the analysis of clones DDRT1, DDRT21D, DDRT24, DDRT25, DDRT35, DDRT36, VL19 and VL21

The results of the BLASTX analysis are presented in Table III. Cadmium exposure causes an increase in the steady-state levels of a several *C. elegans* proteins that are homologous to proteins in the protein databases. Clone VL21 corresponds to a mRNA that encodes the *C. elegans* HSP70F protein precursor. The expression of HSP70 has been shown to increase following cadmium exposure in mammalian cells (Wiegant. F.A. et al. (1994) *Toxicology* 94, 143-159; Hiranuma, K. et al. (1993) *Biochem. Biophys. Res. Commun.* 194, 531-536). This response, however, has not been reported in *C. elegans*. Cadmium exposure also induced the expression of a mRNA that encodes a DNA gyrase homologue, DDRT41. The metal caused a 3-fold increase in the levels of mRNAs DDRT30 and DDRT33D that encode two different *C. elegans* collagens (*col-36* and a predicted cuticle collagen, respectively). It also affected mRNAs that encode proteins that are homologous to a β-adrenergic receptor kinase (DDRT40), pyruvate carboxylase (VL19) and the hypothetical human protein KIAA0174 (DDRT35). BLASTX analysis of the remaining differentially expressed cDNAs did not find significant homologies between the translated sequences and those in the non-redundant GenBank database. Thus, the majority of the cadmium responsive, differentially expressed cDNAs encode novel proteins.

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All documents cited above are hereby incorporated in their entirety by reference, as are all sequences referenced by accession number (e.g., GenBank Accession Number).

One skilled in the art will appreciate from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention.